10/622,026

PATENT SPECIFICATION (11) 1 594 227

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(21) Application No. 45873/77 (22) Filed 4 Nov. 1977 (19)
(31) Convention Application No. 51/144360 (32) Filed 1 Dec. 1976 in
(33) Japan (JP)
(44) Complete Specification Published 30 Jul. 1981
(51) INT. CL.[3] C05F 11/00
(52) Index at Acceptance
C1B 3D5 3DX

(54) A PROCESS FOR PRODUCING AN ACTIVE SUBSTANCE OF THE CYTOKININ SYSTEM

(71) We, NODA SHOKKIN KOGYO K.K. of 121 Shimizu, Noda-shi, Chiba-Pre., Japan; and K.K. IIZUKA KENKYUSHO of 739, Shimizu, Noda-shi, Chiba-Pre., Japan, both Japanese Companies do hereby declare the invention for which we pray that a Patent may be granted to us and the method by which it is to be performed to be particularly described in and by the following statement:-

This invention relates to plant treatment agents.

Trace amount of components such as iron, manganese, silicon, etc. are essential in the culture of higher plants such as rice, soya bean, tomato, potato and chinese cabbage in addition to the three elements of manure of nitrogen, phosphate and potassium. Recently, it was recognized that phytohormone is important for the growth of such plants. Phytohormones are substances which are produced in the plant and it is known that they affect directly or indirectly growth of the stalks, occurrence and formation of roots, formation of leaf and fruits, opening and closing of the stomata, absorption of moisture and formation of flower buds.

At present, five groups such as auxins, gibberellins, cytokinins, abscisins and ethylene are known as phytohormones. These phytohormones may be applied as plant treatment agents, including herbicidal applications, either alone or together with other compounds.

It is known from U.S. Patent 3,961,938 that an aqueous extract of fungi belonging to Basidiomycetes contains germanium and therefore that this extract can be used to promote the growth of plants whose growth is promoted by germanium.

It has now been found that the aqueous extract of Basidiomycetes fungi also contains a component of the cytokinin system and therefore that the utility of this extract as a growth adjusting agent can be extended to plants whose growth is affected by cytokinins but is not promoted by germanium.

The plant treatment agents of the present invention are useful in "cold proofing" plants by inhibiting chlorophyll decomposition, in protecting plants from decay, and in checking disease so that improved yields may be obtained.

According to one aspect of the present invention there is provided a process for the production of an aqueous extract containing an active substance of the cytokinin system which comprises the steps of:-

(i) growing a fungus of the Basidiomycetes family selected from shiitake, hiratake, nameko, shimeji, karawatake or sarunokoshikaki on a solid or liquid nutrient medium;

(ii) adding water to the nutrient medium after the medium has become prevalent with hyphae;

(iii) agitating and mixing the medium and the water; and

(iv) filtering the suspension obtained from step (iii) under pressure.

According to a further aspect of the present invention there is provided a method of adjusting the growth of a plant whose growth is affected by cytokinins but is not promoted by germanium which comprises applying an aqueous extract prepared by the process of the present invention to the leaves or roots of said plant.

Fungi of Basidiomycetes which may be used for this invention, are shiitake (Lentinus edodes (Berk) Sing. C. Shiitake), hiratake (P.ostreatus (Jacq. ex Fr) Quel), nameko (K. nameko (T. Ito) S. Ito et Imai), shimeji (Lyophyllum aggregatum (Schaff. ex Sach), Kawaratake (Coriolus versicolor (L. ex Fr) Quel), & sarunokoshikaki (Polyporaceae). However, the material extracted from hyphae of shiitake has been found to be most active

and is preferred.

The nutrient medium may be, for example, a bagasse nutrient medium consisting of bagasse (remains of sugar cane), which is prepared by mixing bagasse with rice bran in a ratio of 3 to 1, a beet remains nutrient medium consisting of beet remains (remains of sugar beet), which is prepared by mixing beet remains with rice bran in a ratio of 12 to 1, a sawdust nutrient medium which is prepared by mixing sawdust with rice bran in a ration of 3 to 1, or a liquid nutrient medium. However, the main component of a sawdust nutrient medium is sawdust and the growth of the plant is liable to be checked by harmful components, such as resin acid, contained in the sawdust.

Therefore, it is preferred to use a bagasse or a beet remains nutrient medium to make the growth of the plants satisfactory and to cause yield of the plants increase. In particular, bagasse finds no significant commercial use and is mainly burnt so that it is easy to obtain and inexpensive.

If sawdust is used as nutrient medium, it is preferred to refine it beforehand. The refinement may be accomplished by immersing the sawdust in 1% sodium carbonate solution for 24 hours to drive out the harmful components such as resin acid contained in the sawdust through dissolution and by rinsing the sawdust 4 or 5 times with water and removing the water.

A liquid nutrient medium may comprise yeast, ammonium tartrate, an extract of enzyme and boiled juice of bagasse and rice bran. Liquid seed structure is planted in this medium to culture hyphae.

In a preferred method a nutrient medium is sterilized, using a normal method, and a solid seed structure of shiitake (belonging to Basidiomycetes) or liquid seed structure is planted and transferred to the culture chamber to start culture of hyphae. If possible, the nutrient medium is subjected to a temperature change treatment in the culture chamber and provided with air-conditioned equipment to culture hyphae, and after the medium has become prevalent with hyphae or after fruiting bodies are collected, the medium or waste medium is generally crushed into pieces. Water is then added and this mixture is agitated and mixed. Thereafter the suspension obtained is filtered.

According to an analysis, nucleic acid derivative consisting of RNA, sugar alcohols such as inositol and mannitol, eighteen kinds of amino acid, vitamin B and inorganic salts such as magnesium and phosphorous are contained in the extraction liquid extracted by the above mentioned methods. Also, it was verified by a biological examination that this extraction liquid has an active character similar to cytokinin. However, in this extraction liquid, other phytohormones such as abscisinic acid and auxins also seem to be contained.

Fractionation was carried out to separate each active material and the activity of each was measured. The nucleic acid portion and sugar alcohol portion both exhibited an active character but the extraction liquid before fractionation exhibited a higher active character. Accordingly, we believe that the active character of a cytokinin system depends on the correlative effect of each component.

The invention is illustrated in the following Examples:

Example 1

A nutrient medium composed of 90% of bagasse, 5% of rice bran and 5% of nutrient source such as wheat bran was sterilized in the usual way, and a solid seed structure of shiitake was planted in the sterilized medium. The medium was then placed in an air-conditioned culture chamber at a temperature of 18° to 20°C and a relative humidity of 60% to start the culture of hyphae. When the medium was prevalent with hyphae, it was transferred to a high temperature treatment chamber where it was initially heated at a temperature of 32° to 34°C for 24 to 48 hours, and then it was moved to a low temperature treatment chamber to be subjected to a temperature of 5° to 8°C and relative humidity of 85% for 5 to 7 days. The nutrient medium so obtained was moved to the culture chamber.

Hyphae of shiitake started to burst through the surface of the nutrient medium. At this time, the medium was taken out and crushed by means of a crusher into pieces of thumb tip size. The crushed pieces of the medium were placed in a tank, and 5 litres of sterilized water added to 1 kilogram of the crushed medium. The pH was adjusted to 4.5 - 5.0, and the solution agitated for 4 to 5 hours at the temperature of 45° to 50°C, so that the cell membranes of hyphae were broken down by self-digestion and the cell sap of hyphae was dissolved out.

The resulting suspension was then charged into a cloth sack in a filter funnel for filtering under pressure, and the filtrate was re-filtered with a membrane filter to remove bacteria, whereby an extract of hyphae was obtained.

The active character of the extract obtained by the above method was investigated and it was apparent that the extract exhibited the active character of a cytokinin system.

Testing of active character

Experiment 1 - - - - - Leaves of radishes were used.

(a) Examination method

Radishes were cultured outdoors. When the width of the first main leaf reached 5m/m, a disc was hollowed out from the leaves with 5 m/m diameter of cork borer.

The discs were floated on the surface of various concentrated liquid extracts extracted by means of the above method, solutions of kinetin and solutions of gibberellins. The discs were left for 18 hours under artificial light of 2,000 to 2,300 luxes at a temperature of 28°C, and the weight of raw discs, the weight of dried discs and leaf area were then measured and compared.

(b) The results are shown graphically in Figure 1 of the drawings. It is apparent from these that the extract according to the invention gave results similar to those obtained using solutions of kinetin. In particular, the active character obtained with a 1/5000 concentration of the extract was the same as that obtained with 1.0 mg/l. concentration of kinetin solution.

This experiment was carried out in a medicinal plant laboratory of Tokyo Rika University.

The extract was diluted by adding the sterilized water to 1 kilogram of the nutrient medium and by extracting the useful component. In the experiment 5 to 50,000 times diluted solution was used. (These solutions were also used in the following experiments.)

Experiment 2 - - - - - Measurement of active character in growth of paddy (water field rice plant) root.

(a) Examination method

At the bottom of a test tube of 2.5 cm diameter and of 6.0 cm height, absorbent cotton was placed and 5 grains of germinated paddy, Nipponbare, were seeded on it. The extraction liquids of various concentrations were added to the tubes and the tubes were covered with paraffin. Paddies prepared by this method were culture for 7 days at a temperature of 30°C under an artificial light of about 5,000 luxes.

(b) The results are shown in bar graph form in Figure 2.

The experiment was carried out in Iizuka Research Laboratory and the results obtained by observation after keeping the grains for 7 days at a temperature of 30°C. The maximum root length is given for a variety the graph shows the diluted magnification.

According to the above results, it was found that the growth of root was promoted more in the high concentration liquid than in the low concentration liquid. Ratios of values in X_1 section and X_4 section relative to the value in the comparison section (no treatment) were respectively 201% and 137%.

Experiment 3 - - - - - Measurement of active character calculated by decomposition rate of chlorophyll of paddy lamina

(a) Examination procedure

After the paddy seeds, Nipponbare, had germinated, the extraction liquid of various concentration was sprayed on the paddy leaf. On 14th days after seeding, a constant area of leaf was floated on the surface of distilled water, and left for 3 days in a dark room at the temperature of 25°C. The Chlorophyll system was measured to compare with the amount of chlorophyll in the collection time of the laminae, and decomposition rate was shown with percentage.

This examination was conducted in Akita Agricultural Experiment Station.

(b) Examination result

	Concentration of liquid	No. of Treatments	Chlorophyll concentration(1)	Chlorophyll concentration(2)	Decomposition rate
Not treated			0.308	0.067	81.5
Treated by extract	1/250	1	0.298	0.213	28.5
	1/500		0.288	0.203	29.5
	1/750		0.275	0.185	32.7
	1/250	3	0.218	0.203	6.9
	1/500		0.247	0.213	13.8
	1/750		0.243	0.222	8.6

(Note) Concentration (1) - - - Concentration at the time of collection (OD 660 m/u)
Concentration (2) - - - Chlorophyll concentration of the samples which were left for 3 days in a dark room.

Decomposition rate: $100 - (\frac{(Z)}{(T)} \times 100)$

A decomposition restraining effect appeared in all extract treated leaves as compared with the no tratment section. Better results were obtained in case of triple treatment than in case of single treatment.

Accordingly, following results were proved: in Experiment (1) the extraction liquid has a cell expanding effect, in the Experiment (2), an effect promoting redifferentiation of no differentiated organization in the portions such as root or bud, and in the Experiment (3), an aging prevention effect. Such effects are quite similar to the effects due to the active substance of a cytokinin system, so that it is deduced that the active material of a cytokinin system was contained in the extraction liquid.

The above extraction liquid, i.e., active substance extracted from basidiomycetes was used in farm trials and the following results obtained:-

(A) Experiment applied to Chinese Cabbage
Directed by Gunma Gardening Experiment Station
Period August through December of 1975
Procedure

(1) Variety - - - Chiba No. 1
(2) Site - - - - - Gumma Gardening Experiment Station
(3) Treatment and section

Treatment	Remarks
(1) 300 times diluted liquid was sprayed over the leaves	Liquid was sprayed twice when number of main leaves reached 5 and 8 (Sep. 17 and Sept. 25)
(2) 400 times diluted liquid was sprayed over the leaves	
(3) 500 times diluted liquid was sprayed over the leaves	
(4) Not treated	

(Note) Area 1.7 m^2 not repeated

(4) Seeding and cultivation
Seeding - - - August 22 (direct seeding)
Culture area - - - 75 × 45 cm^2
Fertilizer amount - - - N 21.5, P 16, K 18 (Kg/10a)

Result

(a) Growth condition was good in all sections and difference of growth after treatment in each section was not recognised. In the harvest, green colour of the leaf in each section seemed to be more heavy than in the no treatment section.

(b) Yield was higher than in the no treatment section in the order of the 500, 400 and 300 times diluted sections. Main experimental data was as follows.

Investigation of harvest

Treatment	Item	Investigated sample 1	2	3	4	5	Average	Weight ratio
300 times diluted liquid	Longitudinal diameter	28	28	29	29	28	28.4	
	Transverse diameter	17	19	20	17	18	18.2	
	Weight	2,850	3,300	3,600	3,200	3,450	3,280	104
400 times diluted liquid	Longitudinal diameter	28	26	26	30	32	28.4	
	Transverse diameter	18	18	17	19	20	18.4	
	Weight	3,550	3,500	3,000	3,600	3,750	3,480	110
500 times diluted liquid	Longitudinal diameter	29	28	29	30	27	28.6	
	Transverse diameter	19	19	19	20	21	19.6	
	Weight	3,450	3,450	3,450	4,000	3,500	3,570	113
Not treated	Longitudinal diameter	30	27	30	27	27	28.2	
	Transverse diameter	18	17	18	17	18	17.6	
	Weight	3,650	2,900	3,150	2,950	3,050	3,140	100

(Nite) Unit - - - cm, g Investigation date - - - Nov. 15

(B) Experiment applied to potatoes
Directed by Iizuka Research Laboratory (Noda City, Chiba Prefecture)
Period March through June of 1976
Procedure
(1) Variety - - - Danshaku
(2) Site - - - - Iizuka Research Laboratory, farm (Noda City)
(3) Treatment - - 500 times diluted extract was used. Spray over the leaves repeated 2 times. (April 27 and May 7)
Comparison section - - - Water spray
(4) Seeding and cultivation
Seeding - - - March 19
Completing of the number of stalk - - - April 27
Harvest - - - June 15
Fertilizer amount - - - N 13, P 9, K 10 (Kg/10 are)

Result

(a) Growth in each section was smooth and no damage was occured.

(b) The number of potatoes and weights of stalk and leaves were increased in the treatment section as compared with the comparison section.

Main experimental data is as follows:-

	The number of potatoes	Weight	Weight ratio	Weight of stalk	Weight of root
Treated section	8.0	464	133	337	23.8
Comparison section	6.6	350	100	276	16.8

(Gram in average per a stub)

Example 2

A solid seed structure of shiitake was planted in the nutrient medium which was prepared by the same method as in Example (1) and hyphae of shiitake was cultured by means of the method same as in Example (1). When the medium became prevalent with hyphae, the medium was crushed intio pieces of thumb size. The crushed medium was placed in a tank and 5 litre of sterilized water was added to adjust the pH to 4.5 - 5.0. The solution was then agitated for 4 to 5 hours at ambient temperatures (15° to 20°C), to dissolve useful components contained therein, that is, metabolic products of hyphae. The suspension obtained was filtered under pressure using a similar method to that used in the Example (1).

Result of biological assay as in the above Example made clear that a material having the active character of a cytokinin system was contained in the extract.

The same effect was obtained by applying the extraction liquid obtained by the above method to chinese cabbages and potatoes.

Example 3

A sawdust medium combined with 90% of purified sawdust, and 5% of ricc bran, wheat bran, etc. was sterilized by a normal method. Solid seed structure of shiitake was planted in this medium to culture hyphae by means of the method described in Example 1.

After the medium was prevalent with hyphae and immediately before the bursting of fruiting bodies, the medium was crushed into pieces of thumb size, and then, placed in a tank with water added at the rate described above. The water in the tank was heated to 80° to 100°C and the solution agitated for 4 to 5 hours, whereby the metabolic products of hyphae was dissolved in water. The suspension obtained was filtered under pressure by the method used in Example 1.

It was recognized by the biological assay (as in Example 1) that a material exhibiting the active character of a cytokinin system is contained in the extraction liquid.

The extraction liquid obtained by the above method was applied to chinese cabbage and potato and same results as the above experiment was obtained. In addition to the application to the above vegetables, the extraction liquid was also applied to radish. The result was as follows.

(A) Experiment applied to radish
Directed by Iizuka research Laboratory Site Narita City, Chiba Prefecture
Period September through December of Showa 50

Treatment section
(1) Area - - - 10a per variety
(2) 500 times diluted liquid was sprayed over the leaves 3 times.
1st October. - - - At the time of thinning out (when plants had 5 of main leaves)
10th October.
20th October. - - - At the time of corpulence of root portion (Root diameter is about 3 cm)
(3) Spray amount - - - 250 litre per 10 are

Comparison section
(1) Area - - - 2 sections (10 are per section)
(2) No treatment

Seeding and cultivation
(1) Variety - - - (A) Shinmitsuura (B) Miyako
(2) Seeding - - - September 3
(3) Harvest - - - December 9
(4) Fertilizer - - - At first, N 14, P 14, K 14 (60 Kg per 10 are), secondary, N 18, P 15, K 15 (40 Kg per 10 are)

Result

Five examples per each section were selected and examined. Both varieties (Shinmitsuura and Miyako) were different in the root weight, that is, Shinmitsuura increased in weight by 36% and Miyako increased in weight by 20%. In the case of Miyako, the weight of root increased, and in the case of Shinmitsuura, the diameter and length of root increased. Appearance and the inside of the radish were normal.

Main data were as follows:

Harvest investigation (A) Shinmitsuura

		Weight of root (Kg)	Weight of stalk & leaves (Kg)	Maximum root diameter (cm)	Root length (cm)
Experiment section	(1)	3.20	1.0	12.5	43.0
	(2)	3.05	0.9	10.5	59.5
	(3)	3.40	1.2	12.0	49.5
	(4)	2.50	1.9	10.5	45.0
	(5)	2.60	1.2	11.0	43.5
	Average	2.95	1.24	11.3	48.1
Comparison section	(1)	2.55	0.6	9.5	52.5
	(2)	1.90	1.0	9.0	40.5
	(3)	2.15	0.85	9.0	48.0
	(4)	2.55	0.9	9.7	47.0
	(5)	1.65	0.65	8.3	41.0
	Average	2.16	0.80	9.1	45.8

(B) Miyako

		Weight of root (Kg)	Weight of stalk & leaves (Kg)	Maximum root diameter (cm)	Root length
Experiment section	(1)	2.18	0.58	10.1	37
	(2)	2.19	0.58	9.3	43
	(3)	2.26	0.60	10.3	41
	(4)	2.47	0.62	9.9	40
	(5)	2.05	0.56	9.1	43
	Average	2.23	0.59	9.7	40.8
Comparison section	(1)	1.95	0.51	9.5	37
	(2)	1.87	0.49	10.0	37
	(3)	1.88	0.48	9.5	39
	(4)	1.89	0.49	10.5	39
	(5)	2.10	0.55	9.8	39
	Average	1.94	0.50	9.8	38.2

Example 4

In a bagasse medium which was prepared as in Example 1, a solid seed structure of hiratake was planted to culture hyphae by means of the same method. After the medium was prevalent with hyphae and immediately before the bursting of fruiting bodies, the nutrient medium was crushed into pieces of thumb size and the pieces were placed in a tank. Water was added, the mixture heated and agitated and the resulting autodigestion of hyphae was caused to dissolve cell liquid of hyphae.

The result of the biological assay of the extraction liquid made it clear that a material having the active character of a cytokinin system was contained in the extract. However, the active character was inferior to that of hyphae of shittake.

Also, the extraction liquid obtained by the above method was applied to cuttings and the following effects were obtained.

(A) Effect promoting the growth of root of cutting
(a) Experiment of effect to Mukuge (Hibiscus syriacus)
Directed by Iizuka Research Laboratory Site Narita City, Chiba Prefecture
Period March through May 24 of 1976
Number of samples 16 per each section
Treatment
(1) An ear was immersed into the extraction liquid source.
(2) 500 times diluted extraction liquid was used.
(3) Blank - - - sample was immersed into well water for 3 hours.
After the treatment, the sample was inserted into the bed of porous soil containing Aluminium Silicate. (Kanuma soil)

Result:

	Number of appeared root	Rate of appeared root (%)
Blank	2.7	50
Original liquid	9.0	40
500 times diluted liquid	10.7	69

The number of appeared roots was greatly increased by extraction liquid treatment, and the rate of appeared roots also increased in the 500 times diluted liquid section.
(b) Experiment of effect on chryasanthemums
Directed by Iizuka Research Laboratory Site Narita City, Chiba Prefecture
Period May 26 through June 15 of 1976
Number of samples 20 per each section
Treatment
(1) Samples were immersed into 100 times diluted extraction liquid in each case.
(2) Blank - - - Sample was immersed in well water.

Result

	The number of days up to root appearance	The number of appeared root	Rate of appeared root (%)	Maximum length of root (mm)	Weight of dried sample (mg)
Blank	10.0	5.7	63	33.2	2.70
Use of extraction liquid	9.1	13.1	80	36.7	3.58

Example 5

A nutrient medium prepared by the same method as in Example 3 was sterilized using a normal method and a solid seed structure of enokitake was planted in the medium to culture the hyphae. After the medium has become prevalent with hyphae and immediately before the bursting of fruiting bodies, the medium was crushed into pieces of thumb size and placed in a tank with added water in the proportion described above. The solution in the tank was heated to 80° - 100°C and agitated for 4 to 5 hours, whereby the metabolic products of hyphae was dissolved in water. The suspension thus formed was filtered under pressure by the method used in the above Examples.

It was found by the biological assay that a material having the active character of a cytokinin system was contained in the extraction liquid.

The extraction liquid was applied to bean and gladioli and following results were obtained.

(a) Experiment of effect to bean
Directed by Hokkai Siekan Canning Search Laboratory Site Sapporo City
Period May through October of 1975
Seeding and cultivation
(1) Variety - - - Takara bean
(2) Seeding date - - - May 24
Treatment
(1) Single treatment section - - - 500 times diluted solution was sprayed over the leaves (July 14)
(2) Twice treatment section
500 times diluted solution was sprayed over the leaves. (July 14)
300 times diluted solution was sprayed over the leaves. (August 7, at flowering time)
(3) Three times treatment section
500 times diluted solution was sprayed over the leaves. (July 14)
300 times diluted solution was sprayed over the leaves. (August 7)
300 times diluted solution was sprayed over the leaves. (August 27)

Result:

Treatment	Repetition	A	B	Weight of 1000 grains	C	Weight of a root raw	Weight of a root dry	D
		Kg		g		g	g	
Not treated	1	226.7		168.8		10.19	3.89	0.38
	2	180.4		161.6				
	Average	203.6	100	165.2	100			
Once treatment	1	247.1		168.3		11.48	4.74	0.41
	2	244.4		172.9				
	Average	245.8	121	170.6	103			
Twice treatment	1	265.6		167.6		10.06	3.97	0.40
	2	246.0		172.5				
	Average	255.8	126	170.1	103			
Three times treatment	1	247.6		173.0		9.91	3.81	0.38
	2	272.2		177.8				
	Average	259.9	128	175.4	106			

A - - - Weight of fruiting bodies harvested in 10 are area.
B - - - Weight ratio of fruiting bodies relative to the weight of fruiting bodies in no treatment section.
C - - - Weight ratio of 1000 grains of fruiting body relative to the weight of that in no treatment section.
D - - - Ratio of weight of raw root relative to the weight of dry root.

Yield increased greatly by treatment (increase more than about 20% than yield with no treatment) and difference among the treatment sections was such that it suggests that a single treatment is appropriate. The treatment did not appear to affect the root portion to any appreciable extent.

(b) Experiment of effect on gladiolus

Directed by Iizuka Research Laboratory Site Asahi Village, Kashima District, Ibaraki Prefecture

Period April through September of 1974

Seeding and cultivation

Seeding - - - April 19

Fertilizer - - - N 16, P 16, K 16 (150 Kg per 10 are)

Procedure

(1) Area of a section - - - 10 are

(2) Spray amount of extraction liquid per application - - - 250 litre per 10 are

(3) Comparison section - - - Not treated

(4) Experiment section - - - 500 times diluted liquid was sprayed twice at the time of appearance of two leaves and at the time of 30 cm height.

Result:

Ten bulbs were selected in each section for optional measurement. The weight of each bulb was as follows:

No / Section	1	2	3	4	5	6	7	8	9	10	Average
Experiment Section	50	45	45	40	40	50	42	50	52	45	46
Comparison Section	30	40	20	30	30	30	35	37	25	30	28

The difference between the above ground portions was insignificant, but the weight of the bulb portions in the treatment section was 1.6 times those in comparison section.

Example 6

The nutrient medium prepared by means of the method of Example 1 was sterilized, and a solid seed structure of kawaratake was planted in the medium to culture the hyphae. After the medium has become prevalent with hyphae and immediately before the bursting of fruiting bodies, the medium was crushed into pieces. The pieces were then placed in a tank and water was added in the proportions described above. The water in the tank was heated to 45° - 50°C and agitated for 4 to 5 hours. The resulting solution was filtered under pressure as in Example 1.

The biological assay of the extraction liquid obtained demonstrated that a material having the active character of a cytokinin system was contained in the extraction liquid.

The extraction liquid was applied to Taro and the following results were obtained.

Directed by Iizuka Research Laboratory
Period April through November of 1975
Procedure
(1) Variety - - - Aichi wase
(2) Site - - - Noda City, Chiba Prefecture
(3) Treatment and scale

Treatment method	Note
(1) No treatment section	General method
(2) Irrigated soil section	600 litre of 2,000 times diluted solution per 10 are was used at the time of planting.
(3) Seed taro was immersed in the extract solution and the extract was sprayed over the leaves.	Seed taro was immersed in 300 times diluted solution for 30 minutes. 300 times diluted solution was used at the time of 2 and 4 leaves period.
(4) The extract was sprayed over the leaves	300 times diluted solution was used at the time of 2 and 4 leaves.
(5) Seed taro was immersed in the extract solution	Seed taro was immersed in the 300 times diluted extract. Area of a section: $10m^2$ Treatment was repeated.

(4) Seeding and cultivation
Planting - - - April 9
Cultivation area - - - 1 m × 0.3 m
Fertilizer amount - - - N 18, P 14.5, K 13 (Kg per 10 are)
Compost - - - 800 Kg per 10 are

Result (Yield)

Treatment Section		Child taro		Grandchild taro		Great Grand-child taro		Juice	
		Number	Weight	Number	Weight	Number	Weight	Number	Weight
A	1	69	3,180	108	3,130	1	16	178	6,326
	2	81	3,970	99	3,070	-	-	180	7,040
	Average	75.0	3,575	103.5	3,100	0.5	8	179	6,683
B	1	60	3,495	114	4,470	1	16	175	7,981
	2	80	3,960	104	3,545	-	-	184	7,505
	Average	70.0	3,728	109.0	4,008	0.5	8	179.5	7,743
C	1	88	4,030	95	2,590	-	-	183	6,620
	2	76	3,450	103	3,300	-	-	179	6,750
	Average	82.0	3,740	99.0	2,945	-	-	181.0	6,685
D	1	73	3,330	109	3,340	-	-	182	6,670
	2	78	3,610	88	2,635	8	170	174	6,415
	Average	75.5	3,470	98.5	2,988	4	85	178	6,543
E	1	81	4,470	106	3,680	-	-	187	8,150
	2	75	3,850	98	3,260	-	-	173	7,110
	Average	78.0	4,160	98.5	3,470	-	-	180	7,630

A - - - Not treated
B - - - Soil was irrigated
C - - - Seed taro was immersed in the extract solution and the extract was sprayed over the leaves
D - - - The extract was sprayed over the leaves
E - - - Seed taro was immersed in the diluted extract solution.

Summary of results

(1) Good germination was obtained in each section and followed by satisfactory growth.

(2) The number of taro is almost the same with child taro and grandchild taro as in any section. The weight of child taro was superior in the sections in which seed taros were immersed in the extract compared with the other section and the weight of grandchild taro was excellent in the soil irrigation sections and the sections in which seed taros were immersed in the extract. That is, in these sections, corpulence of taros was observed. The result in the sections in which the extract was sprayed over the leaves was almost same as the result in the no treatment section. This is due to the inability of the taro leaf to absorb the useful component of the extract.

Example 7

20 gram of yeast, 2 gram of ammonium tartrate, 2.5 gram of yeast essence, 500 ml of broth of bagasse and rice bran (5 Kg of mixture with bagasse and rice bran in a ratio of 10 to 1) was added into 20 ml of hot water, boiled for 2 hours and the resultant solution filtered through a filter cloth. The filtrate was added to 1 litre of water to prepare liquid nutrient medium and the medium was placed in a jar fermenter having a shaking device for hyphae culture.

A liquid seed structure of shiitake to be cultured was planted in the above liquid nutrient medium to culture the hyphae by shaking for 5 to 7 days. The pH was then adjusted to 4.5 - 5.0, the temperature being raised to about 60°C and the mixture agitated, whereby autodigestion of the hyphae was promoted.

The hyphae (suspension) in the above medium was filtered under pressure using the same method as described in Example 1 to extract the cell sap of hyphae.

As a result of the biological assay of the extract obtained it was found that a material having the active character of a cytokinin system was contained in the extract.

The extract was applied to the bulb of freesia and following results were obtained.

(a) Experiment of effect on freesia

Directed by Iizuka Research Laboratory

Procedure

(1) Variety - - - Golden yellow produced in Hachijo last vear

(2) Site - - - Katashina village, Tone district (800 meters above the sea level)

(3) Treatment and scale

Treatment	Remarks
(1) A section where bulb was immersed in the extract	Bulb was immersed in 5,000 times diluted solution for 30 times.
(2) A section where bulb was immersed and leaves were sprayed with the extract	Bulb was immersed in 5,000 times diluted solution for 30 minutes and the same liquid was sprayed over the leaves twice, on July 4 and August 5.
(3) A section where the extract was sprayed over the leaves	5,000 times diluted solution was sprayed over the leaves twice, on July 4 and August 5.
(4) No treatment section	

100 bulbs per each section were used, no repeat

(4) Seeding and cultivation
Planting - - - April 23
Excavate - - - October 21
Planting interval - - - 5 × 5 cm
Fertilizer amount - - - N 40, P 30, K 30 (Kg per 10 are)

Result:

Investigation of growth and harvested bulbs

Treatment Section	July 4 Length of leaf	July 4 The number of leaves	August 5 Length of leaf	August 5 The number of leaves	A	B	C	D
1	18.0	5.4	35.2	8.0	85	442	5.2	118
2	19.0	5.4	34.8	8.2	84	470	5.6	127
3	19.5	5.8	34.6	8.0	86	447	5.2	118
4	17.0	5.2	31.0	6.6	76	334	4.4	100

1 - - - A section where the bulb was immersed in the extract liquid
2 - - - A section where the bulb was immersed in the extract liquid and the liquid was sprayed over the leaves
3 - - - A section where the liquid was sprayed over the leaves
4 - - - No treatment section
A - - - the number of harvested bulbs
B - - - Weight of harvested raw bulbs
C - - - Average weight of a bulb
D - - - Index of the weight relative to that in the no treatment section

Summary of result:
(1) In each section, 100% of plants germinated.
(2) Growth conditions of the plant whose leaves were sprayed by the extract liquid on July 4 and August 5 were the same, but both were superior to the no treatment section.
(3) The number of bulbs were largely different to each other and in the treatment sections were more rich than that in no treatment section. The average weight of a bulb was relatively heavy in the treatment sections as compared with the no treatment section.

Example 8

The same nutrient medium and seed structure as in Example 1 were used to obtain extracted liquid.

The extracted liquid was applied to water-field rice plant and the following results were obtained.

Experiment procedure

(1) Directed by — The agricultural Experiment Station in Akita Prefecture
(2) Period — May through November of 1975
(3) Variety — Toyonishiki
(4) Seeding date — June 5
(5) Amount of seeds
Seedling (200 gram/case)
Young plant (120 gram/case)

Constitution of experiment sections

No.	Supplied material	Treatment procedure	Diluted concentration	Used liquid amount (cc)	Times of treatment
1	Not treated				
2	Extract	Sprayed over the leaves	250	200	1
3		Same as above	500	200	1
4		Same as above	750	200	1
5	Extract	Sprayed over the leaves	250	200	3
6		Same as above	500	200	3
7		Same as above	750	200	3
8	Extract	Irrigation to soil	250	300	1
9		Same as above	500	300	1
10		Same as above	750	300	1
11	Extract	Irrigation to soil	250	300	3
12		Same as above	500	300	3
13		Same as above	750	300	3
14	Compost liquid	Irrigation to soil	2	400	1
15		Same as above	2	800	1
16		Same as above	2	400	3

(Note) Compost containing micro-organism is used as compost liquid

Result

(1) Result

No.		Height	Leaf's length a	b	c	d	e	Dried weight of 50 samples f	g	h i	j	R/T
	1	12.3	3.6	1.2	4.1	6.0	4.5	1.58	0.58	100	100	36.7
	2	12.4	3.8	0.9	3.3	5.3	5.2	1.54	0.66	98	114	42.9
A	3	11.3	3.4	1.2	4.0	5.8	4.3	1.50	0.68	95	117	46.3
	4	10.6	3.6	1.1	3.8	5.6	4.3	1.90	0.88	123	152	55.0
	5	10.7	3.8	-	3.2	5.3	4.8	1.50	0.70	95	121	46.7
	6	13.1	4.0	-	3.9	6.1	5.1	1.82	0.54	115	93	51.6
	7	11.3	3.6	1.1	4.1	5.9	4.3	1.50	0.68	95	117	45.3
	8	11.9	3.8	-	4.0	6.2	-	1.38	0.70	87	121	50.7
	9	11.1	3.5	1.1	3.8	6.0	5.2	1.38	0.62	87	107	44.9
	10	11.4	3.9	-	3.3	5.3	4.9	1.56	0.78	99	135	50.0
	11	11.5	3.9	-	3.9	5.9	5.5	1.66	0.74	105	128	44.6
	12	12.9	3.8	-	4.2	6.2	4.9	1.84	0.50	117	86	27.2
	13	11.2	3.6	1.1	3.6	5.9	4.7	1.76	0.74	111	128	50.7
	14	13.6	4.0	0.8	2.9	5.6	5.2	1.98	0.86	125	148	48.3
	15	11.3	3.3	1.2	4.4	5.8	4.9	1.46	0.74	92	128	50.7
	16	11.1	3.9	1.3	4.2	5.3	5.4	1.58	0.88	100	152	55.7
	1	28.8	4.2	1.4	4.5	8.1	4.2	9.65	0.95	100	100	9.8
	2	21.7	4.5	1.1	3.6	6.4	5.3	10.60	1.60	110	168	15.0
	3	18.9	4.1	1.2	4.0	7.3	5.8	8.75	1.60	91	168	18.3
	4	21.2	4.0	1.4	4.5	8.0	5.3	8.05	1.10	83	116	13.7
B	5	29.1	4.3	1.3	4.3	8.3	4.9	0.60	1.05	110	111	9.9
	6	20.9	4.3	1.2	3.7	6.4	5.5	7.80	1.00	81	105	12.8
	7	25.3	4.1	1.2	4.3	7.9	4.0	9.10	0.90	94	95	9.9
	8	18.4	4.3	1.2	3.7	6.2	4.2	9.50	1.85	98	195	19.5
	9	21.7	4.3	1.3	3.5	6.2	3.9	7.50	1.10	78	116	14.7
	10	19.8	4.5	1.1	2.9	5.7	4.8	7.45	0.95	77	100	12.8
	11	22.2	4.2	1.1	4.0	6.8	4.2	7.90	0.80	82	84	10.1
	12	25.1	4.4	1.1	3.8	6.9	4.8	9.70	0.90	101	95	9.3
	13	25.1	4.2	1.3	4.2	7.6	3.7	8.90	0.90	92	95	10.1
	14	22.7	4.3	1.0	3.6	6.8	3.9	8.15	0.95	84	100	11.7
	15	19.4	4.5	1.0	3.0	5.8	4.9	7.15	0.90	74	95	12.6
	16	26.2	4.5	1.3	5.0	7.3	4.4	10.15	0.90	105	95	8.9

A - - - Seedling
B - - - Young plant
a - - - The number of leaves
b - - - First leaf
c - - - Second leaf
d - - - Third leaf
e - - - Maximum root length
f - - - Projecting portion over the ground
g - - - Portion under the ground
h - - - Ratio of dried weight of sample relative to that in no treatment section (%)

(2) Concentration of chlorophyll and decomposition ratio of leaf

	Seedling			Young Plant		
	Concentration (1)	(2)	A	Concentration (1)	(2)	A
1	0.308	0.057	81.5	0.458	0.142	69.0
2	0.298	0.213	28.5	0.515	0.288	44.1
3	0.288	0.203	29.5	0.425	0.293	31.1
4	0.275	0.185	32.7	0.483	0.322	33.3
5	0.218	0.203	6.9	0.373	0.358	4.0
6	0.247	0.213	13.8	0.363	0.332	8.5
7	0.243	0.222	8.6	0.447	0.371	17.0
8	0.287	0.222	22.6	0.425	0.379	10.8
9	0.277	0.213	21.3	0.487	0.415	14.8
10	0.243	0.127	47.7	0.487	0.318	34.7
11	0.258	0.203	21.3	0.457	0.423	7.4
12	0.253	0.223	11.9	0.477	0.386	19.1
13	0.287	0.243	15.3	0.465	0.317	31.8
14	0.278	0.203	27.0	0.435	0.338	22.3
15	0.292	0.228	21.9	0.383	0.345	9.9
16	0.308	0.256	16.9	0.468	0.421	10.0

A - - - Decomposition ratio $(100 - \frac{(2)}{(1)} \times 100)$

Concentration (1) - - - Concentration of chlorophyll in sampling (OD 660 m/u)
(2) - - - Concentration of chlorophyll of sample which was immersed in water for 3 days

This experiment was made mainly to observe the low temperature character of the extract. However, it was found from the experimental results as above that the extract is useful to prevent decomposition of chlorophyll. Thus, usefulness of the extract to proof against cold was demonstrated.

Example 9

(a) Effect on wheat which is seeded in spring

A nutrient medium and seed structure same as in Example 1 were used.

The extract was applied to wheat which was seeded in spring and following results were obtained.

Experimental procedure

(1) Directed by Iizuka Research Laboratory
(2) Period April through August of 1976
(3) Site Chureinai Village, Kawanishi District, Hokkaido
(4) Seeding and cultivation
Variety - - - Shunko
Seeding - - - April 28
Harvest - - - August 10
Fertilizer amount - - - N 8, P14.4, K 9.6 Kg/10 are

Treatment and scale:

Treatment method	Note
(1) 300 times diluted liquid was sprayed over the leaves.	When height became 8.3 cm, liquid was sprayed over the leaves. (May 21)
(2) 500 times diluted liquid was sprayed over the leaves.	
(3) 700 times diluted liquid was sprayed over the leaves.	Area of a section - - - 10 are
(4) Not treated section	No repetition

Investigation of growth, height (Average of 20 samples)

Section \ Measurement date	May 21	June 14	June 25
300 times diluted liquid was used	9.7	50.4	73.2
500 times diluted liquid was used	8.5	46.1	75.2
700 times diluted liquid was used	8.0	48.1	75.0
Not treated section	8.5	50.1	78.7

Result

Investigation of yield (Dried weight per 3.3 m^2)

Section	Weight of fruiting body (g)	Grade
300 times diluted liquid was used	530	3
500 times diluted liquid was used	680	2
700 times diluted liquid was used	530	3
Not treated section	370	3

In early days of this period, the temperature was low but the weather was good. In the treatment sections, the stalk and leaves of the plant died later than in no treatment section.

Some of the stalks in no treatment section fell down, but no stalk in treatment section fell down.

In the treatment section, the weight of wheat grain increased remarkably as compared with the no treatment section and in, addition, an increase in the number of wheat grains was observed.

Experiment for the identification of cytokinin component
Directed Dr. Takashi Oriya
Procedure
Sample Extract of edible fungi lyophilized and powdered
Method Thin-layered chromatography was done by applying silica gel (of Merck Co.) as absorption column and n-butanol-acetic acid-water (They are in the ratio 12 : 3 : 5) as developing solvent.

1. (Weight of a callus in carrot)
Weight of a callus of root portion in carrot which is produced in tissue cultures was measured. Cf. Figure 3.

2. (Decomposition and hindrance rate of chlorophyll in a slice of spinach)
After a given section of spinach was suspended in the sample solution and released in darkness for a definite time at constant temperature, its residual weight was measured. Cf. Figure 4.

Measurement of cytokinin component
13 spots appeared on the thin layer were examined each on the above method for measurement of Cytokinin component resulting that, as is seen from Figure 3 and Figure 4, Cytokinin component was found on the spot from 0 to 0.17 and the spot from 0.77 to 0.93 wherein there was a difference upon Cytokinin component heretofore in Rf values.

Judging from the above, it assumed that new material was produced.
Note. In two graphs, BA is an abridgment of "Benzyl adenin".

WHAT WE CLAIM IS:-

1. A process for the production of an aqueous extract containing an active substance of the cytokinin system which comprises the steps of:-
(i) growing a fungus of the Basidiomycetes family selected from shiitake, hiratake, nameko, shimeji, karawatake or sarunokoshikaki on a solid or liquid nutrient medium;
(ii) adding water to the nutrient medium after the medium has become prevalent with hyphae;
(iii) agitating and mixing the medium and the water; and
(iv) filtering the suspension obtained from step (iii) under pressure.

2. A process as claimed in claim 1 wherein water is added to the nutrient medium after the medium has become prevalent with hyphae but immediately before the bursting of fruiting bodies.

3. A process as claimed in claim 1 or claim 2 wherein the nutrient medium is a solid nutrient medium which is crushed before the addition of water thereto.

4. A process as claimed in any of claims 1 to 3 wherein nutrient medium and water mixture is heated during agitation.

5. A process as claimed in any of claims 1 to 4 wherein the pH of the mixture obtained after water addition is adjusted to from 4.5 to 5.0 before agitation and mixing thereof.

6. A process as claimed in claim 4 or claim 5 wherein the mixture is heated at a temperature of from 45° to 50°C for a period of from 4 to 5 hours.

7. A process as claimed in claim 1 substantially as herein described with reference to the Examples.

8. An aqueous extract containing an active substance of the cytokinin system whenever prepared by a process as claims in any of claims 1 to 7.

9. A method of adjusting the growth of a plant whose growth is affected by cytokinins but is not promoted by germanium which comprises applying an aqueous extract as claimed in claim 8 to the leaves or roots of said plant.

10. A method as claimed in claim 9 substantially as herein described.

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Printed for Her Majesty's Stationery Office, by Croydon Printing Company Limited, Croydon, Surrey, 1981.
Published by The Patent Office, 25 Southampton Buildings, London, WC2A 1AY, from which copies may be obtained.

1594227 COMPLETE SPECIFICATION

2 SHEETS *This drawing is a reproduction of the Original on a reduced scale*

Sheet 1

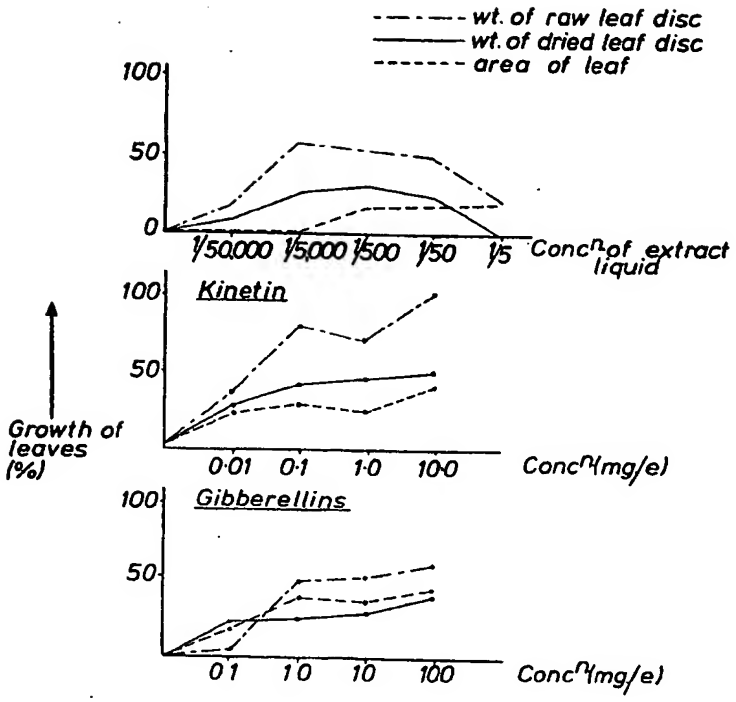

Fig. 1

1594227 COMPLETE SPECIFICATION
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Sheet 2

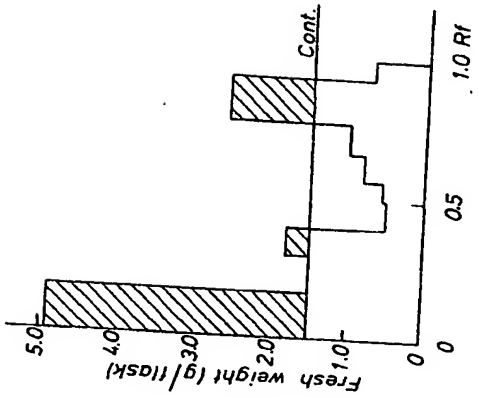

Fig. 4

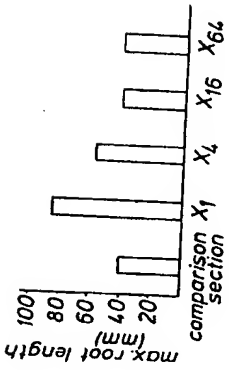

Fig. 2

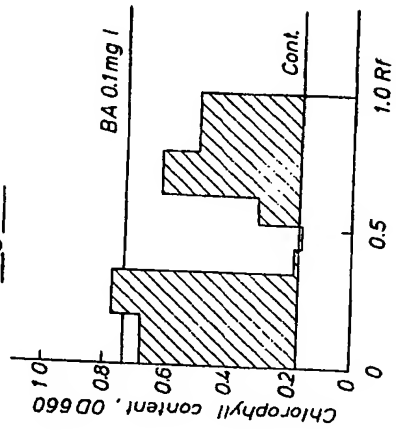

Fig. 3